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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The central aim of this research is to provide information on the neurochemical processes that underlie the generation and entrainment of mammalian circadian rhythms. The studies are centered around the newly-developed in vivo brain microdialysis technique for assessing the daily patterns of neurotransmitter release in the suprachiasmatic nuclei (SCN) of freely-behaving hamsters. During the funded period, this approach has yielded several new findings related to the activities of serotonergic and excitatory amino acid systems in the SCN. Specifically, it was found that: 1) there are daily variations in extracellular concentrations of 5-HIAA and glutamate in the SCN, with highest levels occurring at night; 2) the daily release pattern of glutamate, but not serotonin, in the SCN is circadian in nature; 3) the rhythm in glutamate measured in SCN microdialysate is based upon a non-synaptic, calcium-dependent mechanism and does not appear to be directly linked to the expression of locomotory behavior; and 4) serotonergic transmission suppresses glutamate in SCN microdialysate, an effect possibly related to a modulatory effect of serotonin on glutamate release in the SCN. This also may be closely related to our finding that serotonin blocks light-induced Fos protein expression in the SCN.

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STUDY OF SCN NEUROCHEMISTRY USING IN VIVO MICRODIALYSIS IN THE CONSCIOUS BRAIN: CORRELATION WITH CIRCADIAN ACTIVITY RHYTHMS.

J.D. Glass, U.E. Hauser, W. Randolph, S. Ferriera, and M.A. Rea[1] Dept. Biol. Sci. Kent State University, Kent, OH 44242; [1]Armstrong Lab. (CFTO) Brooks AFB, TX AFOSR 89-NL-071.

A. **ABSTRACT.** The central aim of this research is to provide information on the neurochemical processes that underlie the generation and entrainment of mammalian circadian rhythms. The studies are centered around the newly-developed in vivo brain microdialysis technique for assessing the daily pattern of extracellular neurotransmitter release in the suprachiasmatic hypothalamus of freely-behaving Syrian and Siberian hamsters. This approach has yielded several new findings related to the activities of serotonergic and excitatory amino acid systems in the SCN. Specifically, it was found that: 1) there are daily variations in extracellular concentrations of 5-HIAA and glutamate in the region of the SCN, with highest levels occurring at night; 2) the daily release pattern of glutamate, but not serotonin, in the SCN region is circadian in nature; 3) the rhythm in glutamate measured in SCN microdialysate is based upon a non-synaptic, calcium-dependent mechanism and does not appear to be directly linked to the expression of locomotory behavior; and 4) serotonin suppresses glutamate in SCN microdialysate, an effect possibly related to a modulatory effect of serotonin on glutamate release in the SCN.

B. **SUMMARY.** A number of neurotransmitter substances found in the SCN have been implicated in the regulation of circadian pacemaker function. Of these, serotonin and the excitatory amino acid transmitter, glutamate have received considerable attention, because of their marked effects on circadian rhythms demonstrated in vivo and in vitro. Despite this information, the roles of these transmitters in the various aspects of pacemaker activity, including entrainment, rhythm generation and efferent linkage for expression of overt rhythms are not understood. The focus of the present studies, therefore, was to assess the daily patterns of activity of the serotonergic and glutaminergic systems in the region of the SCN in freely-behaving hamsters using in vivo microdialysis.

Analyses of microdialysates from the SCN region of the Syrian hamster under a 14L:10D photoperiod (LD; n=6) revealed significant daily variations in the extracellular concentrations of 5-hydroxyindoleacetic acid (5-HIAA), glutamate and aspartate. In each case, highest levels occurred during the dark phase and lowest levels during the morning. There was not significant daily variation in the non-transmitter amino acid, glutamine. The nocturnal peak of 5-HIAA (127% of the daily mean) occurred within 2 h after lights-off, during the initial bout of wheel-running. The peak release of glutamate (239% of the daily mean) occurred later during the dark phase (0200 h -0300 h) and there were two peaks of aspartate release at 0100 h - 0300 h and 0700 h - 0800 h. Peaks in glutamate and aspartate were not consistently associated with bouts of wheel-running. Only 8% of nocturnal peaks in 5-HIAA release identified in 6 animals coincided with a peak in glutamate, and the extent of discordance between peaks in 5-HIAA and glutamate is shown in a representative profile (Fig. 1). In sharp contrast to the rhythmic

pattern of 5-HIAA under LD, the apparent diurnal variation in extracellular concentration in this serotonin metabolite was not evident after 2 wk exposure to constant dim light (<0.4 lux; DD). The apparent daily rhythm in glutamate persisted under these conditions, however, with peak levels occurring during subjective night.

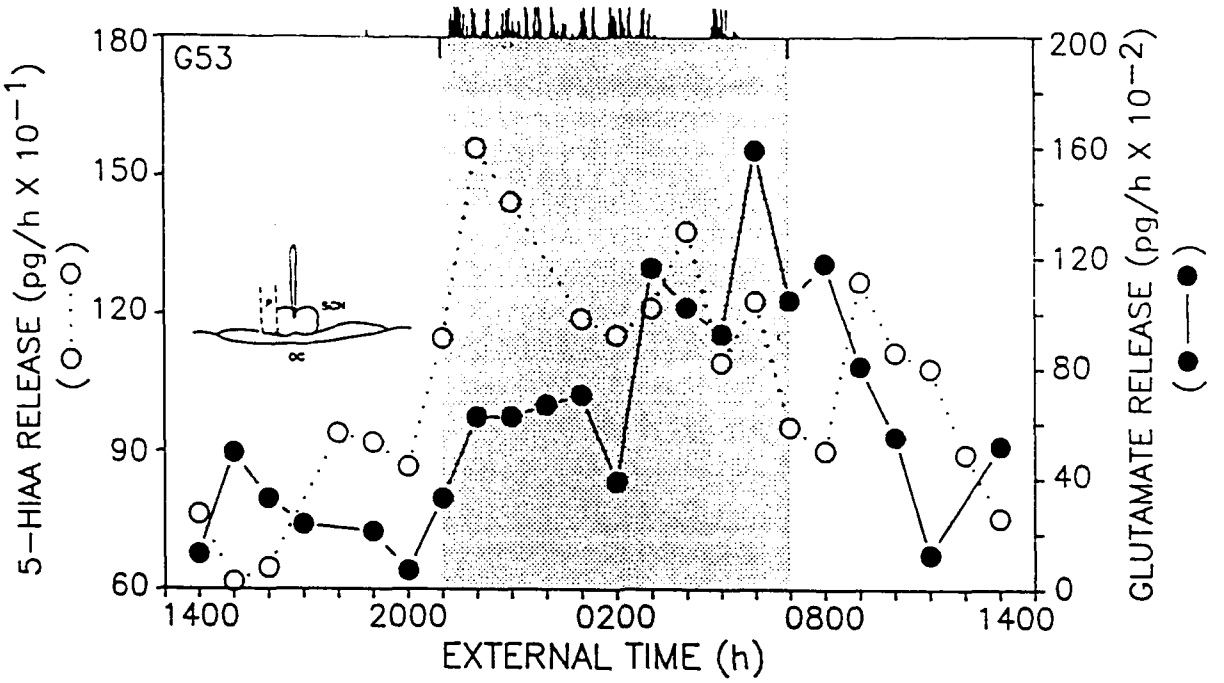

FIGURE 1. Representative daily profile of 5-HIAA and glutamate release in a hamster maintained under LD 14:10. Shown above is an actogram of the animal's wheel-running activity monitored over the sampling period. Also shown is a diagrammatic representation of probe placement relative to the SCN. Stippling denotes dark phase.

The effects of a number of pharmacological agents were examined to assess the significance of the apparent daily variation in extracellular 5-HIAA and glutamate in the SCN. In initial studies (Glass et al., Neuroendocrinology, 1992 [in press]; the minimal number of animals used in each group described below was 5) it was shown that direct application of tetrodotoxin (TTX) to the SCN decreases 5-HIAA at night, but had little effect during the day (42±8 vs. 12±5% suppression, respectively; $p<0.05$). Also, systemic administration of the serotonin release inhibitor, citalopram, decreased 5-HIAA to a greater extent during the night than during the day (21±4 vs. 8±3% suppression, respectively; $p<0.05$). Moreover, the metabolism of exogenous serotonin to 5-HIAA is significantly greater at night ($p<0.05$). Together, these results are strong evidence for a nocturnal activation of serotonin release and metabolism in the SCN. The nocturnal release of glutamate was not significantly affected by TTX ($p<0.36$ vs. baseline), but was stimulated 9-fold by Kcl depolarization in a calcium-dependent manner. These results suggest that the rhythm in glutamate measured in SCN microdialysate is based upon a non-synaptic, calcium dependent mechanism. The nocturnal occurrence of the glutamate peak, together with its persistence under DD, point to a non-RHT release of

glutamate contributing to peak output.

The temporal discordance between nocturnal peaks in 5-HIAA and glutamate measured in SCN microdialysates led us to hypothesize a dynamic interaction between these systems. This idea was tested, in part, by assessing the effect of localized application of serotonin-creatinine sulfate salt or the serotonin agonist, quipazine via the dialysis probe on extracellular glutamate. Both treatments significantly lowered glutamate in SCN microdialysate, with maximal suppression of 49.5±9.5 and 48.0±6.0%, respectively (both $p<0.05$ vs baseline). Control treatment with creatinine sulfate had no effect. These results indicate that serotonergic transmission is inhibitory to the release of glutamate in the SCN region. The purpose for such a modulatory effect of serotonin on glutamate release is speculative, but may be related to the timing of the apparent circadian rhythm in extracellular glutamate measured in SCN microdialysate. Theoretically, timing of a nocturnal glutamate peak could be important to circadian function, as exogenous glutamate applied to the SCN has been shown to phase shift the activity rhythm under DD and LL.

The suspected role of serotonin in modulating SCN pacemaker function was investigated further by assessing the effect of serotonergic activation on light-induced, RHT (possibly glutamate) mediated immediate-early gene (c-fos) expression in the SCN. Hamsters maintained under LD received either an i.p. injection of the serotonin agonist quipazine (25 mg/kg) or saline 30 min before exposure to a 30 min light flash at CT 19.5. Treatment effects were quantified by counting the number of cells expressing fos-like immunoreactivity (FLI) in the SCN section containing the largest number of immunostained cells. Treatment with quipazine caused a striking (60%) reduction in FLI positive cells ($p<0.004$; $n=12$), with the most pronounced inhibitory effect in the ventrolateral aspect of the SCN. If the assumption that glutamate relays photic information from the RHT to the SCN is correct, it is possible that quipazine inhibited fos expression by suppressing release and/or postsynaptic action of glutamate. Experiments involving intra-SCN application of serotonin agonists are underway to assess this possibility.

C. PUBLICATIONS.

1. MANUSCRIPTS SUBMITTED FOR PUBLICATION.

Rea, M.A., Ferriera, S.A., Randolph, W. and Glass, J.D. In vivo microdialysisof the suprachiasmatic nuclei: Evidence for a nocturnal increase in glutamate release. Submitted, Neuroendocrinology.

Glass, J.D., Hauser, U.E., Blank, J.L., Selim, M. and Rea, M.A. Differential timing of amino acid and 5-HIAA concentration in the suprachiasmatic hypothalamus. Submitted, American J. Physiology.

Selim, M., Glass, J.D., Hauser, U.E. and Rea, M.A. Serotonergic inhibition of Light-induced Fos protein expression and extracellular glutamate in the suprachiasmatic nuclei. Submitted, Brain Research.

2. PUBLISHED PAPERS (OR IN PRESS).

Glass, J.D., Randolph, W., Ferreira, S.A., Rea, M.A., Hauser, U.E., Blank, J.L. and De Vries, M. Diurnal variation in 5-HIAA output in the suprachiasmatic region of the Siberian hamster assessed by in vivo microdialysis: Evidence for nocturnal activation of serotonin release. Neuroendocrinology, 1992 (reprint enclosed).

Glass, J.D., Hauser, U.E., Randolph, W., Rea, M.A. and De Vries, M. In vivo microdialysis of 5-HIAA and glutamic acid in the hamster SCN. American J. Zoology, 1993 (in press).

Glass, J.D., Hauser, U.E., Randolph, W. Ferriera, S.A., Rea, M.A. Study of SCN neurochemistry using in vivo microdialysis in the conscious brain: Correlation with circadian activity rhythms. J. Biological Rhythms (AFOSR Suppliment), 1993.

3. PUBLISHED ABSTRACTS.

Rea, M.A., Blank, J.L., Ferreira, S.A., Terrian, D. and Glass, J.D. In vivo microdialysis of amino acids in the suprachiasmatic nuclei. Soc. Neurosci. 1989 abst. # 293.10.

Ferreira, S.A., Randolph, W., Rea, M.A. and Glass, J.D. Effects of melatonin on the neurochemistry of the suprachiasmatic nucleus. Soc. Neurosci. 1990 abst. # 317.17.

Ferreira, S.A., Randolph, W., and Glass, J.D. In vivo evidence for a diurnal rhythm in SCN serotonergic activity in the Siberian hamster. Soc. Neurosci. 1991 abst. # 264.16.

Glass, J.D., Hauser, U.E., Blank, J.L. and Rea, M.A. In vivo assessment of 5-HT and amino acid transmission in the SCN: Correlation with overt circadian rhythms. Soc. Neurosci. 1991 abst.# 15.5.

Hauser, U.E., Van den Beukel, I. and Glass, J.D. Daily profiles of in vivo 5-HIAA and glutamate release in the amygdalaof the female Syrian Hamster. Soc. Neurosci. 1992 abst.# 366.9.

D. PROFESSIONAL PERSONNEL ASSOCIATED WITH THE RESEARCH.

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E. INTERACTIONS

i. Paper presentations.

Soc. Neurosci, Oct, 1989
Soc. Neurosci, Oct, 1990
Cleveland St. Univ., May 1990
Northeastern Ohio Col. Med., Oct 1990
Kent State Univ., Oct 1990
Am. Soc. Zool., Dec. 1990
Soc. Neurosci. Nov, 1991 (2 presentations)
Soc. Neurosci. Nov. 1992
AFOSR program Review, Nov., 1992

i.i. Consultative and Advisory Functions.

1. Since the beginning of the funded program of research, we have been actively collaborating with Dr. Michael A. Rea at the Neuroscience Laboratory, Brooks AFB, Tx. The nature of the interaction involves various methodologies, including microdialysis and immunocytochemical procedures to assess the role(s) of glutamate and serotonin in SCN function. We have several papers and abstracts that are accepted or submitted containing work from our collaboration.

2. Since April 1990 we have interacted with Dr. Mark Rollag at the Dept. Anatomy, Uniformed Services University, Bethesda Md. Together, we are studying the pharmacokinetics of melatonin in the SCN region.

F. NEW DISCOVERIES. N/A

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Diurnal Variation in 5-Hydroxyindole-Acetic Acid Output in the Suprachiasmatic Region of the Siberian Hamster Assessed by in vivo Microdialysis: Evidence for Nocturnal Activation of Serotonin Release

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Abstract
In vivo brain microdialysis was used to characterize the daily pattern of 5-hydroxyindole-acetic acid (5-HIAA) release in the region of the suprachiasmatic nuclei (SCN) in freely behaving male Siberian hamsters housed under 16L:8D. A marked diurnal variation in the concentration of extracellular 5-HIAA was apparent, with peak levels (147 ± 5% of the daily mean; $p < 0.05$) occurring 2–3 h after lights-off. Smaller nocturnal rises in extracellular 5-HIAA were observed in the posterior hypothalamus and preoptic area (128 ± 4 and 123 ± 8% of the daily mean, respectively; both $p < 0.05$ vs. average daytime levels). Tryptophan loading increased 5-HIAA in SCN microdialysates by 44 ± 6%, and this response was enhanced by localized perfusion with tetrodotoxin (TTX; 5 μ*M*). Localized applications of KCl (150 m*M*) or veratridine (100 μ*M*) decreased 5-HIAA by 62 ± 5 or 49 ± 11%, respectively. The effect of KCl was not significantly affected by specific calcium channel blockers. Perfusion with TTX markedly decreased SCN 5-HIAA during the dark phase, but had little effect during the light phase (42 ± 8 vs. 12 ± 5% suppression, respectively; $p < 0.01$). Addition of serotonin (3 μ*M*) to the perfusate significantly stimulated 5-HIAA output. This treatment increased the release of 5-HIAA more during the dark than during the light phase (61 ± 8 vs. 25 ± 5%, respectively; $p < 0.01$). Taken together, these results are evidence that: (1) there is increased TTX-sensitive (synaptic) release of serotonin at night that could contribute to the nocturnal rise in the extracellular concentration of 5-HIAA in the SCN; (2) clearance (uptake and/or metabolism) of extracellular serotonin in the SCN is higher at night; (3) 5-HIAA release in the SCN of the Siberian hamster is similar to that reported for other areas of the brain in other species, and (4) variations in 5-HIAA may, under certain physiological conditions, reflect changes in the extracellular concentration of serotonin.

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The suprachiasmatic nuclei (SCN) are the principal neural apparatus for the generation and maintenance of circadian rhythms in physiology and behavior [1, 2]. The SCN is comprised of cells that exhibit a self-generating pacemaker activity [3, 4] and is richly innervated by amino acid, peptidergic and serotonergic projections [5, 6]. The intrinsic pacemaker activity of the SCN system is entrained by light, which serves to couple the timing of internal circadian rhythms to the prevailing 24-hour light-dark (LD) cycle [7]. Input to the SCN pacemaker is provided via direct afferent visual projections from the retina [8] as well as from indirect connections from the lateral geniculate nuclei via the intergeniculate leaflet [9]. In addition, there is a major projection from the mesencephalic median and dorsal raphe nuclei [10, 11].

Observations that the SCN have one of the highest concentrations of serotonin in the brain and that serotonin projections provide the densest innervation of the SCN other than from the direct and indirect retinal projections [10] have led to speculation that serotonergic innervation is part of the mechanism underlying SCN function. The importance of serotonergic innervation is supported by numerous studies on the effects of manipulating central serotonin levels on behavioral and endocrine rhythms. For example, depletion of serotonin by electrolytic lesions of the raphe nuclei disrupts the expression of free-running motor activity rhythms during exposure to constant dark and constant light [12–14]. Whole-brain depletion of serotonin using the serotonin neurotoxin 5,7-dihydrotryptamine alters the timing of the active period relative to the LD cycle [15] and delays the establishment of the blood corticosterone rhythm in rats [16]. Depletion of brain serotonin by parachlorophenylalanine has been shown to markedly suppress motor activity rhythms [17] and to attenuate daily rhythms of feeding and corticosterone [17, 18]. Increasing the concentration of hypothalamic serotonin with the monoamine oxidase inhibitor clorgyline significantly delays the onset of the activity rhythm under LD and lengthens the period of free-running activity under constant dark [19, 20]. Finally, treatment of SCN slice preparations with serotonin agonists or antagonists have been shown to induce dramatic phase shifts in the circadian pattern of spontaneous electrical activity [4, 21].

Diurnal fluctuations in uptake [22–24] and sensitivity [25] to exogenous serotonin in the SCN point to a time-dependent functional interaction between the pacemaker system and serotonergic activity. A daily rhythm in release of serotonin may be involved in this process, since in vivo techniques including voltammetry [26], and push-pull cannulation [27] have revealed a striking daily variation in the extracellular concentration of the principal serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the SCN. The significance of the 5-HIAA rhythm is not certain, however, because there is a large body of evidence suggesting that extracellular 5-HIAA may be derived primarily from intracellular metabolism of unreleased serotonin [4, 28–33]. Further, the in vivo methods used to measure 5-HIAA to date cannot resolve serotonin release in regions as small as the SCN. The nocturnal peak of 5-HIAA in the SCN, therefore, may be related to factors affecting serotonin metabolism, and not release.

The present study was undertaken to examine the nature of the rhythmic discharge of 5-HIAA in the region of the SCN to further understand the action of serotonin in the circadian pacemaker system. The Siberian hamster *(Phodopus sungorus)* was adopted as the animal model because this species has been widely used in studies of the SCN in regulating circadian and circannual rhythms [34, 35]. We also wished to compare the pharmacology of 5-HIAA release in the SCN to that reported in other brain regions. Extracellular 5-HIAA was measured using the technique of brain microdialysis, which has been used extensively in studies on 5-HIAA release [30, 36].

Materials and Methods

Animals

Adult male Siberian hamsters ranging in age from 8–15 weeks and weighing approximately 40 g were used in the present study. The animals were raised in the Department of Biological Sciences Vivarium at Kent State University in a temperature-controlled room (23 °C) under a 16L:8D photoperiod with lights-on at 08.00 h Eastern Standard Time and light intensity of 250 lx. Food (Purina Rodent Chow, Ralston Purina, St. Louis, Mo., USA) and water were provided ad libitum.

Microdialysis Probes

Probes of a concentrically arranged design were used in the present study. Each probe was constructed from hemicellulose dialysis tubing allowing the passage of molecules $\leq$ 6 kD (230 µm outside diameter; Spectra-Por, Fisher) glued to a 2.6-cm-long 26-gauge stainless-steel outer cannula (Small Parts, Miami, Fla., USA). The distal end of the dialysis tubing extended 1.5 mm from the outer cannula and was capped with epoxy glue, which provided a dialyzing tip length of 0.8–1.0 mm. The inner cannula consisted of a 3.5-cm length of fused silica tubing (o.d. = 0.145 mm; Polymicro Technologies, Phoenix, Ariz., USA) with its end beveled at a 45° angle. Inflow and outflow connections were made from 3.5-cm lengths of 0.51 and 0.25 mm inside diameter microline tubing, respectively (Cole Parmer, Chicago, Ill., USA).

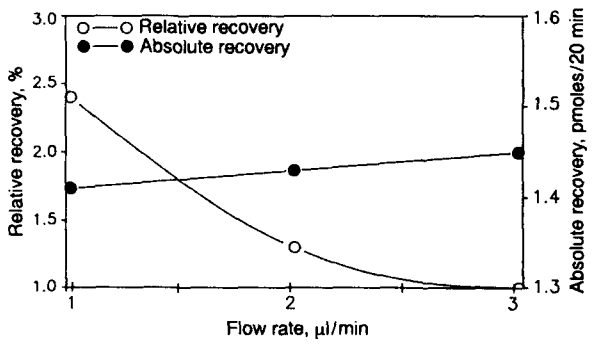

Fig. 1. Characteristics of in vitro 5-HIAA uptake by the dialysis probes used in the present study. Absolute recovery is the total mass of 5-HIAA taken up by the probe over the collection interval. Relative recovery, an index of probe efficiency (ratio of [5-HIAA] in dialysate/[5-HIAA] in the outside solution) was approximately 2.4% at a flow rate of 1 µl/min.

Probes were perfused with artificial cerebrospinal fluid (ACSF: NaCl 126.5 m*M*, KCl 2.4 m*M*, $CaCl_2$ 1.1 m*M*, $MgCl_2$ 0.85 m*M*, $NaHCO_3$ 27.5 m*M*, KH_2PO_4 0.5 m*M*, Na_2SO_4 0.5 m*M*, and glucose 5.9 m*M*, pH 7.5) at a flow rate of 1 or 2 µl/min. The dialysate exited the probe via a 10-cm length of microline tubing that led to a 250-µl vial clipped to the probe assembly. The time for fluid to reach the collection vial from the tip of the probe was 12 min at a flow rate of 1.0 µl/min. Relative recovery (used as the index of probe efficiency) was determined in vitro as the ratio of [5-HIAA] in the dialysate/[5-HIAA] in the outside solution at a flow rate of 1 µl/min at 37 °C.

Dialysis Probe Implantation

Under sodium pentobarbital anesthesia (Nembutal 50 mg/kg), hamsters received a probe implant with the dialysis membrane tip stereotaxically placed in or near the lateral margin of the SCN to avoid damage to the nuclei [coordinates: anterior/posterior (AP) = –0.15 mm from bregma, lateral (L) = +0.30 mm from the midsagittal suture and horizonal (H) = –7.40 mm from the dura, with head level]. The second and third groups of animals received a probe implant in the preoptic area (AP = 0.0 mm, L = +0.3 mm, H = –4.9! mm) or posterior hypothalamus (AP = 1.5 mm, L = +0.3 mm, H = – 5.80 mm). The probe was anchored to the skull with 3 stainless-steel screws and cemented in place with dental acrylic. Animals were treated with Combiotic (7,500 U; Pfizer, New York, N.Y., USA) and allowed 48 h to recover prior to experimentation. After this time, they were placed in a circular (25 × 20 cm) clear plastic cage and connected to a syringe pump [CMA 200, Bioanalytical Systems (BAS) West Lafayette, Ind., USA] by microbore Teflon tubing (BAS) and an overhead liquid swivel (Instech Laboratories, Plymouth Meeting, Pa., USA). At the end of the study animals were killed by Nembutal overdose and the site of implantation evaluated from 10-µm-thick frozen sections mounted and stained with cresyl violet.

High-Performance Liquid Chromatography Electrochemical Detection

A high-performance liquid chromatograph (HPLC) equipped with an amperometric electrochemical detector (Bioanalytical Systems, Model 200A) was used to determine the concentrations of 5-HIAA as described previously in this laboratory [37]. A 20-µl aliquot of dialysate was injected directly into a 3-µm C-18 column (100 × 3.2 mm, phase II, BAS). Working standards were obtained from Sigma Chemical Co. (St. Louis, Mo., USA) and prepared in 0.10 *N* perchloric acid. The mobile phase consisted of 9.45 g monochloroacetic acid, 3.6 g NaOH, 0.25 g Na_2EDTA and 0.2 g octane sulfonic acid in 1 liter of purified distilled water. The mobile phase was adjusted to pH 3.1 with NaOH and filtered before use. Tetrahydrofuran (2 ml) was added to the mobile phase after filtration. The flow rate of the mobile phase was 1.0 ml/min, and a glassy carbon electrode, set at a potential of 750 mV relative to an AgCl reference electrode, was used as the detector. Under these conditions, sensitivity, i.e. the minimal amount of 5-HIAA producing a signal twice that of background, was ≤2.5 pg.

Experimental Protocol

Dialysates were collected in 10 µl 0.1 *N* perchloric acid and immediately assayed by HPLC. For assessment of the daily pattern of 5-HIAA release, hourly samples were collected continuously over 24 h at a flow rate of 1 µl/min. Collection of samples began at midday, and the animals were maintained under 16L:8D photoperiod, with nighttime samples collected under dim red light (≤0.4 lx). In experiments involving the administration of pharmacological agents to the SCN and/or alteration of the ionic content of the ACSF, 20-min samples were collected at a perfusion rate of 2 µl/min. Sample collection started 2 h before each treatment in order to establish stable baseline conditions. Each treatment was standardized with respect to time of day.

Reagents and Drugs

The effects of a number of pharmacological agents were examined to characterize the relationship between extracellular 5-HIAA and serotonin release. *L*-tryptophan (Sigma; 5.0 mg/ml) was dissolved in normal saline at room temperature for intraperitoneal injection. Tetrodotoxin (TTX; 5 µ*M*), serotonin (300 µ*M*), veratridine (100 µ*M*) and calcium channel blockers (diltiazem HCl 200 µ*M*, verapamil 200 µ*M*, cinnarizine 15 µ*M* and flunarizine 12 µ*M*; Sigma) were dissolved in ACSF and administered through the dialysis probe. When high [KCl] (150 m*M*) was administered, [NaCl] was reduced to 28.5 m*M* to maintain osmolarity of the perfusate. Reagents and solvents used in preparation of ACSF and for HPLC analysis were of HPLC grade.

Statistics

Output of 5-HIAA was expressed as pg/sampling interval. Inter-animal variation in baseline concentrations of 5-HIAA were standardized by expressing treatment values as a percentage of baseline output. Baseline was calculated as the mean of three 20-min pretreatment samples. Analysis of variance followed by a multiple range test (Student-Newman-Keuls), or paired t-test was used for the assessment of diurnal fluctuations in 5-HIAA release and effects of pharmacological manipulations. The level for statistical significance was $p < 0.05$.

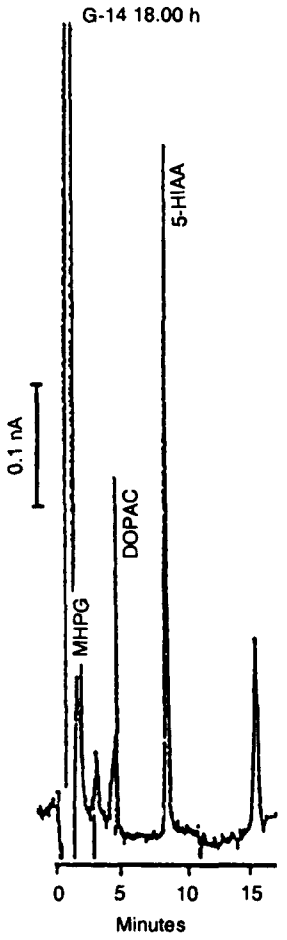

Fig. 2. Representative HPLC chromatogram of dialysate from the region of the SCN collected in a freely behaving hamster approximately 2.5 days after dialysis probe implantation (see Materials and Methods for chromatography conditions).

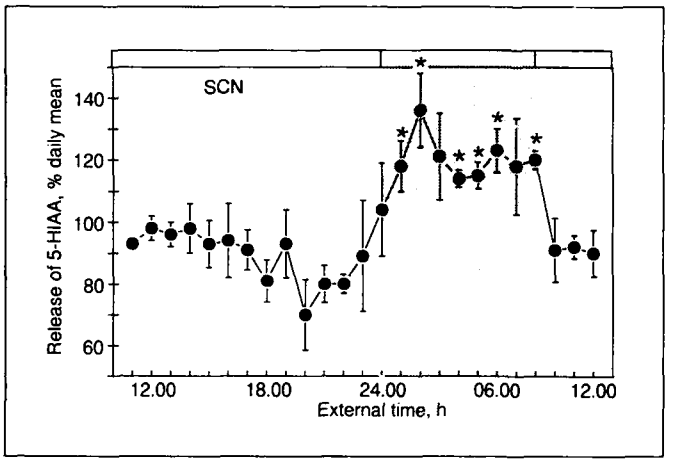

Fig. 3. Averaged release of 5-HIAA ± SEM for 6 hamsters with a dialysis probe in the region of the SCN. Stippling indicates dark phase. *$p < 0.05$, a significantly higher hourly rate of 5-HIAA release compared to the mean light-phase level.

Results

Microdialysis HPLC Characteristics

The relative in vitro recovery of 5-HIAA by the probes was 2.4 ± 0.2%, estimated from 25 probes tested in $2.6 \times 10^{-5} M$ 5-HIAA at 37 °C at a perfusion rate of 1 µl/min. Recovery was inversely related to the rate of perfusion (fig. 1). Baseline concentrations of 5-HIAA in samples collected from the region of the SCN ranged from 2 to 10 pg/µl of dialysate (see fig. 2 for representative chromatogram). A 20-µl sample injection volume was used for HPLC-electrochemical analyses of brain microdialysates. Response of the HPLC-electrochemical detector system was linear in the range of 5–200 pg 5-HIAA, and the lower limit of sensitivity of the system for reproducible integration of peaks was 2.5 pg. Long-term sampling (≥ 24 h) did not deplete brain 5-HIAA levels and did not interfere with eating, sleeping or motor activities.

Daily Pattern of 5-HIAA Release

Output of 5-HIAA exhibited a diurnal rhythm in samples obtained from probes in or near the lateral SCN. A nadir occurred prior to lights-off (20.00 h), while peak levels occurred 2 h after lights-off (02.00 h, fig. 3). Release of 5-HIAA dropped at lights-on to levels similar to the previous morning. Timing of the nocturnal peak rise in 5-HIAA output was somewhat variable between animals (fig. 4), however, in each case this was temporally associated with the initial bout of motor activity logged shortly after lights-off. Later nocturnal peaks in 5-HIAA were not always associated with bouts of motor activity. The peak rate of 5-HIAA release during the dark period increased to 147 ± 5% of the mean daytime level ($p < 0.05$).

An apparent diurnal rhythm in release of 5-HIAA was also evident in the preoptic area and posterior hypothalamus, with peak levels occurring at lights-off (128 ± 4 and 123 ± 8% of the mean daytime level, respectively; $p < 0.05$, fig. 5). In both of these regions, the peak rate of 5-HIAA release occurred 2 h earlier than in the SCN, after which time there was a gradual decline to basal lev-

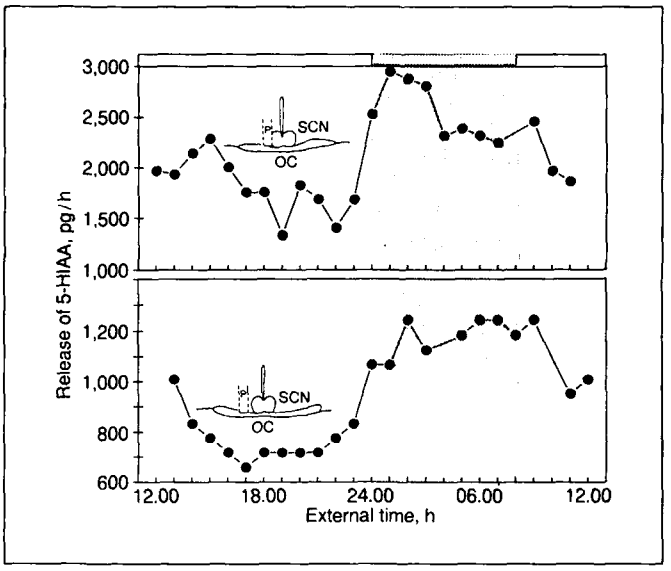

Fig. 4. Representative profiles of the daily pattern of 5-HIAA release in the region of the SCN in 2 hamsters. The probes were situated in or near the lateral SCN. Stippling indicates dark phase. P = Probe; OC = optic chiasma.

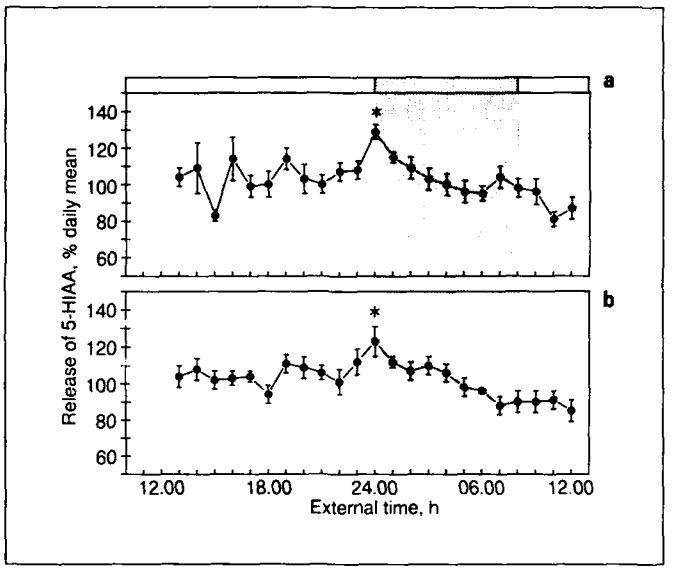

Fig. 5. Averaged release of 5-HIAA ± SEM for hamsters with a probe in the preoptic area (**a**, n = 7) or posterior hypothalamus (**b**, n = 6). Stippling indicates dark phase. *p < 0.05, significantly higher hourly 5-HIAA release compared to mean light-phase level.

els. In both regions the amplitude of the nocturnal increase in 5-HIAA was less than in the SCN.

Effects of Pharmacological Agents on 5-HIAA Release in the SCN

Perfusion of the SCN region with 150 m*M* KCl via the microdialysis probe for 60 min (starting at 14.00 h) suppressed 5-HIAA release by 62.5 ± 9.4 % ($p < 0.01$, fig. 6). This decline was reversed by return to normal ACSF and was not significantly affected by calcium channel blockers (N-type, cinnarizine and flunarizine and *L*-type, verapamil and diltiazem) in calcium-free ACSF (fig. 6). Perfusion with the sodium channel activator veratridine for 20 min (starting at 12.00 h) reduced 5-HIAA release by 54.5 ± 13.5% ($p < 0.01$, fig. 7). Intraperitoneal injection of *l*-tryptophan (starting at 14.00 h) caused a 42.2 ± 6.6% increase in 5-HIAA release, with peak levels occurring 60 min after injection ($p < 0.01$, fig. 8). This effect was significantly enhanced by localized 1-hour perfusion with TTX commencing 60 min after the tryptophan injection ($p < 0.02$, fig. 8).

The effect of TTX on 5-HIAA release varied with the LD cycle. Perfusion with 5 μ*M* TTX for 60 min during the light phase (starting at 09.00 h) had little suppressive effect on 5-HIAA release (fig. 9). In contrast, this treatment caused a maximal 42.4 ± 9.2% reduction in 5-HIAA during the dark phase (starting at 03.00 h; $p < 0.01$ vs. light-phase effect). This suppression of 5-HIAA by TTX continued throughout the course of the experiment. Localized application of serotonin had a significant effect on 5-HIAA release that also varied with the LD cycle. Perfusion with 3 μ*M* serotonin for 120 min during the light phase (starting at 09.00 h) caused a small but significant rise in the concentration of 5-HIAA in microdialysate (25.2 ± 4.8%; $p < 0.01$ compared to baseline, fig. 10). In contrast, perfusion with serotonin during the dark phase (starting at 03.00 h) caused a much greater stimulation of 5-HIAA (60.8 ± 7.9%; $p < 0.002$ vs. light-phase effect).

Discussion

Serotonergic transmission is thought to play a critical role in the regulation of mammalian circadian rhythms including those of body temperature, feeding, sleep and endocrine secretion [14, 17, 18, 25]. The disruptive effects of lesions of the raphe nuclei or their projections [12–14] and pharmacological manipulations of serotonin synthesis and degradation [15–20] on behavioral and endocrine rhythms underscore the importance of serotonin. Although the basis for the action of serotonin in the SCN has not yet been established, diurnal rhythms in hypothalamic serotonin

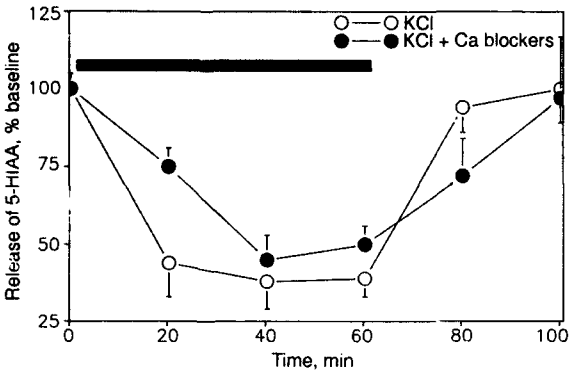

Fig. 6. Effect of localized perfusion of 150 m*M* KCl on 5-HIAA in SCN dialysates (n = 6). KCl was administered for 60 min (▬) by inclusion in the ACSF. Coperfusion with a mixture of specific calcium channel blockers (n = 5, see Materials and Methods for details) did not significantly interfere with the suppressive effect of KCl. Vertical lines are SEM.

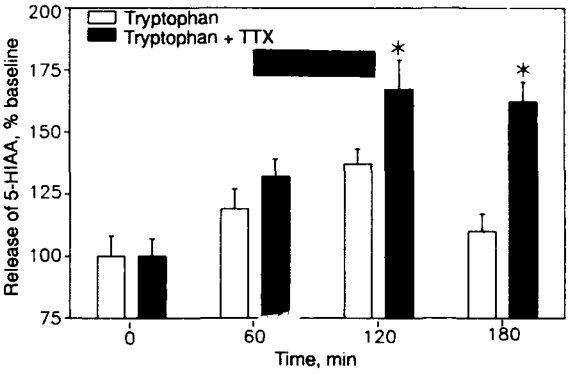

Fig. 8. Effect of intraperitoneal injection of *l*-tryptophan on 5-HIAA in SCN dialysate. Localized perfusion with TTX for 1 h (▬) significantly increased the release of 5-HIAA at 2 and 3 h after injection vs. tryptophan treatment alone. For both groups n = 5. Vertical lines are SEM. *p < 0.05.

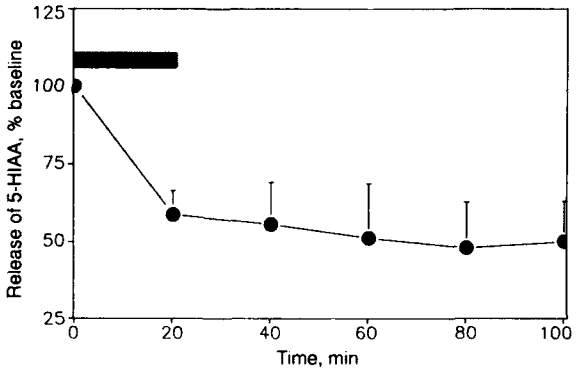

Fig. 7. Effect of localized perfusion with the sodium channel activator veratridine on 5-HIAA in SCN dialysates (n = 5). Veratridine was administered for 20 min (▬) by inclusion in ACSF. Vertical lines are SEM (baseline SEM is smaller than symbol).

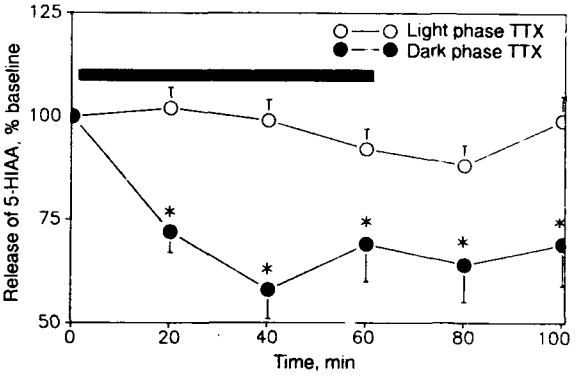

Fig. 9. Effect of localized 60-min perfusion with TTX (▬) on release of 5-HIAA in the SCN region. This treatment had little effect during the light phase, but markedly suppressed 5-HIAA during the dark phase. For both groups n = 6. Vertical lines are SEM. *p < 0.05, time-of-day differences.

concentration [25, 38, 39], uptake [23, 24] and electrophysiological effects [4, 21, 40] have been observed. Moreover, striking diurnal fluctuations in the extracellular concentration of the principal serotonin metabolite, 5-HIAA, have been documented in the mammalian SCN [26, 27]. Collectively, these results give strong evidence that modulation of responsiveness of serotonin targets in the SCN together with regulated timing of serotonin release are important to the regulation of pacemaker function.

The present investigation is the first to apply the technique of in vivo brain microdialysis to explore the pattern of 5-HIAA release in the SCN region. Our results are consistent with those of previous studies using in vivo voltametric [26] and push-pull cannula [27] methodologies

in the rat SCN, in which marked daily variation in 5-HIAA release was observed. In each study, extracellular 5-HIAA release rose during late subjective evening and peaked near the time of lights-off. Peak release of 5-HIAA in the rat and hamster was temporally associated with the initial bout of nocturnal locomotory activity. Smaller nocturnal peaks in 5-HIAA release were also observed in microdialysates from regions rostral (preoptic area) and caudal (posterior hypothalamus including the dorso- and ventromedial nuclei) to the SCN. This is indicative of a generalized activation of serotonergic activity throughout the hypothalamus, and concurs with reports of nocturnal 5-HIAA peaks in the paraventricular nucleus [34], ventral [25] and lateral [41] regions of the hypothalamus. A nocturnal rise in serotonin has also been reported in the posterior hypothalamus [42].

Despite the robust and stereotypic nature of the apparent diurnal rhythm of 5-HIAA release in the mammalian SCN, its functional relationship to serotonergic synaptic activity is still unclear. This uncertainty has arisen from limitations in the current in vivo methodology for resolving the release of extracellular serotonin within the SCN as well as from numerous pharmacological investigations indicating that 5-HIAA may be an unreliable indicator of serotonin release [29–33, 43]. Collectively, these studies have shown that when the release of serotonin is elevated by neuronal depolarization using agents such as KCl, 5-HIAA is reduced [30, 43, 44]. Also, inhibition of spontaneous serotonin release using the sodium channel blocker TTX has little effect on 5-HIAA levels, and 5-HIAA is not significantly affected by direct application of serotonin reuptake inhibitors [30, 32, 43, 44]. Findings such as these have been taken as evidence that extracellular 5-HIAA levels reflect primarily the intraneuronal metabolism of an unreleased pool of serotonin. Thus, the diurnal rhythm of 5-HIAA seen in the SCN may reflect alterations in the metabolism of unreleased serotonin (i.e. availability of tryptophan, and kinetics of tryptophan hydroxylase and/or monoamine oxidase [23] rather than changes in serotonergic synaptic transmission per se.

Two original lines of evidence from the present study indicate that the nocturnal peak in 5-HIAA is reflective of increased metabolism and release of serotonin. First, our observation that localized application of the sodium channel blocker TTX to the SCN significantly reduced microdialysate concentrations of 5-HIAA during the dark phase, but had little effect during the light phase, indicates that the 5-HIAA measured at night arises from an enhancement of sodium channel-dependent (synaptic) release of serotonin. Concordance between the 47% elevation in 5-HIAA at night and the 42% inhibition of 5-HIAA by TTX suggests that synaptically released serotonin is the principal source of the nocturnal increase in 5-HIAA. The relative lack of effect of TTX during the day indicates that most of the extracellular 5-HIAA pool during this period arises from the metabolism of unreleased serotonin. Our second finding that 5-HIAA release is significantly enhanced by the addition of serotonin to the perfusate is direct evidence that increased extracellular serotonin may, under some conditions, be reflected in increased concentrations of 5-HIAA in microdialysate. Importantly, the significantly higher rate of conversion of exogenous serotonin to 5-HIAA during the dark phase indicates that the clearance of serotonin is enhanced at night. This finding is consistent with observations that the hypothalamic uptake of serotonin is higher at night [23, 24] and with experiments involving the use of [^{3}H]tryptophan to estimate rates of serotonin turnover in hypothalamic slices, where there were nocturnal increases in the ratio of labelled 5-HIAA/serotonin as well as serotonin release [29].

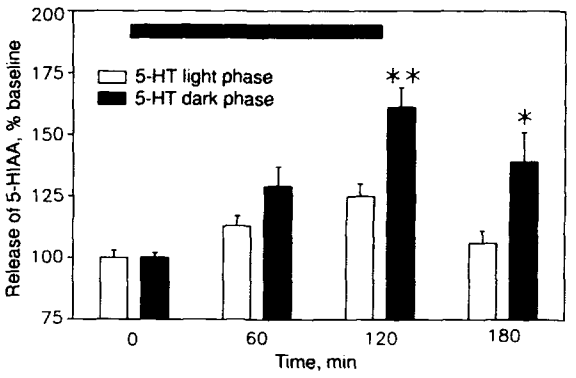

Fig. 10. Effects of localized 2-hour perfusion with serotonin (▬) on 5-HIAA in SCN dialysate. This treatment increased the release of 5-HIAA during the light and dark phases, with a significantly enhanced effect during the dark phase. For both groups n = 5. Vertical lines are SEM. *p < 0.05, **p < 0.01, time-of-day differences.

Although localized application of TTX reversibly reduces the spontaneous release of serotonin [30, 32, 43], there is considerable variability in the literature on the effect of this treatment on 5-HIAA release. For example, applicaton of TTX during the day has little effect on 5-HIAA release in the caudate putamen [30] and SCN (present study), but significantly reduces 5-HIAA in the lateral hypothalamus [43]. On the other hand, administra-

tion of TTX during the night suppresses 5-HIAA in the SCN (present study) but not in the posterior hypothalamus [42]. A reason for this variability may be the difference in dosages used in the studies because 5-HIAA is suppressed by the higher (5–10 μ*M*, [43]) but not by the lower (0.5–1.0 μ*M*, [30, 42]) concentrations of TTX in perfusion fluid. Another important consideration is a time-of-day difference in the effect of TTX on 5-HIAA release resultant from the apparent diurnal variation in serotonergic activity.

The inhibitory effect of KCl-induced depolarization on 5-HIAA release in the region of the SCN is similar to that reported for the caudate putamen [30], cortex, thalamus and anterior hypothalamus [44] as well as lateral hypothalamus [43]. The reason for the suppression of 5-HIAA by KCl is unclear, since this treatment dramatically increases extracellular serotonin [30]. One possibility is that the high concentration of K^+ ions reversibly impairs serotonin reuptake or monoamine oxidase activities. Our experiments showing that localized perfusion with the depolarizing agent, veratridine, produces a similar (albeit longer-lasting) inhibition of 5-HIAA suggests, however, that this is not the case. Another possibility is the proposed inhibitory effect of membrane depolarization on 5-HIAA efflux [44], although the lack of a compensatory overshoot of 5-HIAA after KCl removal weakens this contention. Our demonstration that calcium channel blockers do not significantly attenuate this effect points further to the involvement of a calcium-independent mechanism not related to serotonin release.

The significant elevation of 5-HIAA in SCN microdialysates induced by tryptophan is consistent with that seen in rat cortical microdialysates [45]. These results indicate that tryptophan has a widespread effect on extracellular 5-HIAA release in the brain. As the nocturnal rise in extracellular 5-HIAA may be due to dietary tryptophan intake, it is possible that feeding could be an important stimulus for the nocturnal increase in serotonergic activity. This hypothesis was, in part, tested in the present study by comparing the (suppressive) effect of TTX on the nocturnal 5-HIAA peak to the effect of TTX on 5-HIAA release stimulated by tryptophan loading. In marked contrast to the inhibition of 5-HIAA by TTX an night, TTX had a stimulatory effect on the tryptophan-induced increase in 5-HIAA. The reason for this stimulation is not clear, but could reflect a higher efficiency of metabolism of the newly synthesized, unreleased serotonin. Noteably, the qualitatively different response between the two treatments indicates that, in contrast to the cortex, where TTX suppressed the effect of tryptophan loading [45], the onset of the 5-HIAA peak in the SCN may not be triggered by tryptophan intake. Consistent with this idea is that the evening rise in 5-HIAA in the present study occurred before lights-off, when the hamsters are asleep, and that circadian variation in blood and brain tryptophan levels are not correlated with serotonin turnover [38].

In conclusion, the daily pattern of 5-HIAA release in the male Siberian hamster SCN is comparable to that measured in the rat using push-pull cannulation [27] or voltametry [26]. The increased inhibitory effect of TTX on 5-HIAA release during the dark phase, compared to the light phase, is evidence that there is a nocturnal increase in serotonergic transmission within the SCN. Furthermore, the increased rate of production of 5-HIAA from exogenous serotonin during the dark phase points to an enhanced capacity for serotonin clearance during this period. The suitability of the microdialysis procedure for studies on SCN neurochemistry is demonstrated by: (*1*) the biochemical viability of nerve endings and pathways at the probe site as evidenced by their ability to take up and metabolize exogenous serotonin, and (2) the animals' expression of normal overt behaviors throughout the experiments. This methodology is currently being used to study the nature of the daily rhythm in 5-HIAA under free-running conditions and to explore the linkage between serotonergic activity and behavioral state.

Acknowledgments

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